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The Morphological and Genetic Distinctness of *Botrychium minganense* and *B. crenulatum* as Assessed by Morphometric Analysis and RAPD Markers

LINDA M. SWARTZ[1] and STEVEN J. BRUNSFELD[2]
Department of Forest Resources, University of Idaho, Moscow, ID 83844-1133

ABSTRACT.—Two species of *Botrychium* subgenus *Botrychium* (moonworts, Ophioglossaceae), *Botrychium minganense* Victorin and *B. crenulatum* W. H. Wagner, can sometimes be confused in the field, even by experts, because of their reduced morphology. *Botrychium minganense* can imitate *B. crenulatum*, which is more rare. They are afforded different degrees of protection on Federal lands, making the distinctness of these species a question of management, conservation, and systematic interest. The purpose of this study was to compare a morphometric analysis of these two species with an analysis of DNA markers from the same individuals, and to assess their distinctness under each method. Collections were made in Washington, Oregon, Idaho, and Montana from seven populations of *B. crenulatum* and 18 populations of *B. minganense*. Each plant was measured, emphasizing characters cited by authors in the original species descriptions. Canonical variate analysis performed on SAS separated the samples into two species groups with 32% overlap. RAPD genetic markers revealed more genetic variation than has previously been documented in moonworts. UPGMA cluster analysis of the similarity of RAPD profiles showed well-defined *B. minganense* and *B. crenulatum* clusters, but no distinct clusters within *B. minganense* that could be correlated with its morphological variability. Small samples of the moonwort species *B. lunaria* and *B. simplex* included for comparison also formed distinct clusters. *Botrychium crenulatum* had seven unique RAPD bands, and identification of *B. crenulatum* could be confirmed or ruled out with markers from one or two RAPD primers. Both *B. crenulatum* and *B. lunaria* have been suggested as possible diploid parents of tetraploid *B. minganense*. All RAPD markers absent in *B. crenulatum* but present in *B. minganense* were also present or polymorphic in *B. lunaria*, supporting *B. lunaria* as a possible parent. One very small population of *B. minganense* showed a monomorphic RAPD profile, consistent with inbreeding, but all other populations had multiple genotypes. Some plants of *B. minganense* clustered most closely with plants from populations up to 400 km away, suggesting that variation may be introduced into populations by occasional colonization by spores from distant sources.

Species of *Botrychium* subgenus *Botrychium* (moonworts, Ophioglossaceae) are an enigmatic part of the temperate flora, notable for their small size, reduced morphology and difficult identification. Several moonwort species in North America are listed as sensitive or rare because of small and/or few known populations. Small populations are more vulnerable to extirpation, whether from natural stochastic events or human activities. Understanding the threat to species requires accurate information on numbers of individuals and

[1] Current address: USDA Forest Service, Hiawatha National Forest, St. Ignace Ranger District, 1798 West Highway 2, St. Ignace, MI 49781.
[2] Corresponding author.

populations. If species intergrade morphologically, questions can arise not only about actual numbers of individuals and populations but also about species boundaries and the genetic distinctness of each species.

All moonworts are relatively small and bear a single leaf with a fertile segment (sporophore) and sterile segment (trophophore) each season from an underground bud. They are notoriously hard to find, especially in thick vegetation. As increasing emphasis has been focused on rare plants in recent decades, more concentrated searches have extended the known ranges of common *Botrychium* species and provided material from which 13 new species have been described since 1980. Several of these are endemic to western North America: *B. crenulatum* W. H. Wagner, *B. echo* W. H. Wagner, *B. lineare* W. H. Wagner, *B. montanum* W. H. Wagner, *B. paradoxum* W. H. Wagner, *B. pedunculosum* W. H. Wagner, *B. pumicola* Coville, and *B. pinnatum* H. St. John (the latter two described in 1900 and 1929, respectively). Distinguishing moonwort species in the field often depends on subtle differences in phenology, color, texture, proportions of the parts of the single leaf, and dissection of the pinnae. Such species, poorly morphologically differentiated but evolutionarily distinct, have been called cryptic species (Stebbins, 1950; Paris, Wagner, and Wagner, 1989; Hauk and Haufler, 1999). Although species differences may be subtle, some species are also quite variable among regions, among sites, and even within the same site (e.g., Wagner and Lord, 1956). One of those species is *B. minganense* Victorin.

This study was initiated in response to the practical need to distinguish between *B. crenulatum* and *B. minganense*. These two species have been confused in the western United States by many botanists (Zika, 1992). Although both have been listed as "sensitive" in the past by National Forests in the Pacific Northwest Region (Region 6) and the Northern Region (Region 1), *B. minganense* has been delisted in Region 6 in response to the discovery of many more populations, while *B. crenulatum* retains its official status as rare. Species designation affects management options where *B. crenulatum* occurs. The documented distribution of *B. crenulatum* is the mountain states of the American west (Arizona, California, Idaho, Montana, Oregon, Nevada, Utah, Washington, and Wyoming), whereas *B. minganense* is widespread in the western mountains and across northern North America (Wagner and Wagner, 1993).

Botanists have employed both lumping and splitting approaches to the confusing variability of *B. minganense*. *Botrychium minganense* has been interpreted by many authors as a variety of *B. lunaria* (L.) Sw. (see Wagner and Lord, 1956 for discussion). In *Flora of the Pacific Northwest* (Hitchcock and Cronquist, 1973), only five moonworts are recognized, and the taxon to which *B. minganense* keys is called *B. lunaria* var. *onongadense* (Underw.) House. Cronquist said that *B. minganense* is "...morphologically scarcely separable from diploid var. *onongadense*..." and considered it conspecific with *B. lunaria* (Gleason and Cronquist, 1991). *Botrychium minganense* is a currently accepted taxon (International Taxonomic Information System database http://www.itis.usda.gov/plantproj/itis, April 15, 2000; Kartesz, 1994). In the most

recent treatment of North American moonworts (Wagner and Wagner, 1993), *B. minganense* is reported to be sometimes misidentified as *B. dusenii* of South America. It is also easily confused with *B. lunaria* (Wagner and Lord, 1956; Farrar, 1998), *B. ascendens* (Zika, 1992; Farrar, 1998), *B. pallidum* (Zika, 1992), *B. spathulatum* (Zika, 1992), and *B. crenulatum* (Wagner and Lord, 1956; Lellinger, 1985; Wagner and Devine, 1989; Zika, 1992; Farrar, 1998). Zika (1992) described *B. minganense* as "treacherously variable". Just as *B. minganense* was recognized as an independent species from the more widespread and common *B. lunaria*, so too were *B. pallidum* and *B. spathulatum* formerly confused with *B. minganense*. Both Wagner and Wagner (1988), and Wagner (1994) have suggested that *B. minganense* may represent a species complex.

Unlike *B. minganense*, *B. crenulatum* is more constant in form when well developed, but as with any moonwort, the identity of small plants can be ambiguous. *Botrychium minganense* can approach the form of *B. crenulatum* closely. Wagner and Wagner (1981) state that some of the collections on which the original description of *B. crenulatum* was based were originally identified as *B. lunaria* var. *minganense*.

Botrychium crenulatum is diploid ($2n = 90$, F. S. Wagner, 1993), whereas *B. minganense* is tetraploid ($2n = 180$ Wagner and Lord, 1956; but see Hauk and Haufler, 1999). Many fern species, however, have races with different ploidy levels (e.g. *Asplenium trichomanes*, Wagner et al., 1993), and ideally, additional evidence of genetic differences would be employed to separate species (for discussion, see Gastony and Windham, 1989).

Molecular techniques are well suited to clarify problems of cryptic species. Hauk (1995) used *rbcL* sequences in a phylogenetic analysis of 20 species of *Botrychium* subgenus *Botrychium*. Hauk found that four samples of *B. minganense* (from Michigan, Colorado, and Ontario) shared identical sequences, along with *B. paradoxum* and *B.* ×*watertonense*, and lacked the single synapomorphies that distinguished the simplex and campestre subclades of the "simplex-campestre" clade. *Botrychium crenulatum* formed a separate clade with *B. lunaria*, identical in sequence to the United States *B. lunaria* sample, and well separated from the "simplex-campestre" clade by a total of nine substitutions.

In contrast to the *rbcL* data, which did not distinguish *B. crenulatum* from *B. lunaria*, isozymes differentiated *B. crenulatum* from all others (Farrar, 1998; Hauk and Haufler, 1999). Among the sampled diploids *B. crenulatum* was most similar to *B. lunaria*, but their genetic identity (Nei, 1978) was only 0.53 (Hauk and Haufler, 1999). *Botrychium minganense* possessed the highest variability of the western moonworts (Hauk and Haufler 1999, Farrar 1998), but neither study inferred the variation to be indicative of species-level differentiation within *B. minganense*.

Random Amplified Polymorphic DNA (RAPD) markers, a type of genetic fingerprint, have revealed a level of genetic variation useful for distinguishing populations and sometimes species, and typically possess more variation than isozymes (for reviews, see Bachmann, 1997; Crawford, 1997). RAPD has been particularly useful in assessing variation in rare plants because, as a PCR-based

technique, it requires only small tissue samples, fresh or dried. DNA markers such as RAPD may provide important information for critically assessing morphometric analyses in taxonomically confusing groups. Morphometric analysis can suffer from a circular logic in which a taxon exhibits a certain range of morphologic variation because of assumptions made in the assignment of specimens to that taxon. Assigning specimens based on genetic markers can provide more robust morphometric insights, as has been shown in a variety of studies (e.g., Hardig et al., 2000).

Thus, the goals of this study are 1) to determine the genetic distinctness of *B. minganense* and *B. crenulatum*, on the basis of RAPD markers 2) to document patterns of genetic variation within *B. minganense* and *B. crenulatum*, on the basis of RAPD markers, and 3) to assess quantitatively the morphological differences between plants of *B. minganense* and *B. crenulatum* classified on the basis of genetic markers.

Methods

Collections.—Samples were collected from seven populations of *Botrychium crenulatum* and 18 populations of *B. minganense* in the states of Washington, Oregon, Idaho, and Montana (Table 1). Within this region populations were chosen to include a full range of habitats and geography. Plants with morphology intermediate between the two species were collected when found, and small plants were collected as well as large, well-developed ones to represent a full spectrum of the morphology found in each population. Plants were collected throughout the spatial extent of each site. Sample sizes are given in Table 1. In addition, two populations of *B. lunaria* and one of *B. simplex* E. Hitchcock (both subgenus *Botrychium*) were collected to provide a larger sample of species level molecular comparisons. The ecological associations of *B. minganense* and *B. crenulatum* were quite different in different parts of their ranges. *Botrychium crenulatum* in Washington is sometimes found in somewhat wetter and more open habitats than *B. minganense*, but the large populations sampled for this study were all growing under a *Thuja plicata*/mixed conifer canopy on subirrigated ground. By contrast, the Goofy Springs, Oregon, population was growing in heavy graminoid cover in an opening on seepy ground; the Stewart Creek, Montana, site was a wet mowed roadside and ditch; and at Lapover Ranch, Oregon (the one site on private land), *B. crenulatum* and *B. minganense* were growing together in grass cover under *Pinus contorta* with no spring evident. In Washington, *B. minganense* was found almost exclusively under riparian *Thuja plicata* stands with depauperate understory, but one sampled population (Mill Gate) came from an herbaceous mountain meadow. The association with *Thuja plicata* stands may be an artifact of the circumstance that moonworts have mainly been searched for in association with proposed timber sales. In Oregon, the sampled *B. minganense* populations were all under forest canopy open enough to support a luxuriant shrub and/or herb layer, except Dusty, which was from a wet meadow.

TABLE 1. Collection locations of *Botrychium* used in this study. Collections from some sites were segregated under more than one collection number. Where more than one analyzed species occurred at a single site, each species is listed as a population with an identifying letter (m = *B. minganense*, c = *B. crenulatum*, L = *B. lunaria*). Vouchers are deposited in ID.

Species	Site (no. of plants)	Site abbreviation	Location	Voucher
B. minganense	Watson Point (2)	Watson	OR, Wheeler Co.	Swartz 387
	Flowery Trail (6)	Flowery	WA, Stevens Co.	Swartz 393
	Kelsey Creek (6)	Kelsey	MT, Lincoln Co.	Swartz 394
	Rock Bottom (7)	Rock	ID, Boundary Co.	Swartz 398
	PWB Deer (7)	Deer	ID, Boundary Co.	Swartz 399
	Wenatchee Ford TSHE (7)	WenT	WA, Chelan Co.	Swartz 401A
	Wenatchee Ford RUPA (5)	WenR	WA, Chelan Co.	Swartz 401B
	Devil's Club Creek (7)	Devil	WA Chelan Co.	Swartz 402
	Mill Gate (17)	Mill, MillB	WA, Chelan Co.	Swartz 403, Swartz 453
	Aladdin 1 (6)	Aladdin1	WA, Stevens Co.	Swartz 414
	Bulldog Cabin (5)	Bulldog	WA, Stevens Co.	Swartz 420
	Poison Springs (7)	Poison	OR, Grant Co.	Swartz 425
	m Hodgson Creek (7)	mHodgson	WA, Ferry Co.	Swartz 466
	m Rd. #9576 (6)	mRd9576, ManleyX	WA, Ferry Co.	Swartz 468, Swartz 486
	m Manley Creek (15)	mManley	WA, Ferry Co.	Swartz 470
	La Grande 32 (6)	LaG32	OR, Union Co.	Swartz 504
	Shady Camp (7)	Shady	OR, Wallowa Co.	Swartz 506
	Dusty (6)	Dusty	OR, Union Co.	Swartz/ Riley 508, Swartz/ Yanskey 509
B. crenulatum	Goofy Spring (7)	Goofy	OR, Crook Co.	Swartz 388
	Stewart Creek (5)	Stewart	MT, Flathead Co.	Swartz 396
	Okanogan Cabin (7)	OKCabin	WA, Okanogan Co.	Swartz 404
	Aladdin Blowdown (7)	Aladdin	WA, Stevens Co.	Swartz 427
	Deadman Creek (5)	Deadman	WA, Ferry Co.	Swartz 445
	c Hodgson Creek (7)	cHodgson	WA, Ferry Co.	Swartz 467
	Lapover Ranch (10)	Lapover	OR, Wallowa Co.	Swartz 507
B. lunaria	L Rd. #9576 (5)	LRd9576	WA, Ferry Co.	Swartz 469
	L Manley Creek (7)	LManley	WA, Ferry Co.	Swartz 471
B. simplex	La Grande Meadow (5)	LGMead	OR, Union Co.	Swartz 505

Plants were collected by snipping them off at ground level to avoid disturbing the roots and the next year's below-ground bud. This procedure is not believed to have a significant negative impact on survival (Johnson-Groh and Farrar, 1996; Montgomery, 1990). Where possible, plants were collected after they had shed spores. Plants were pressed, individually numbered, color photocopied, and digitally imaged before grinding for DNA extraction.

The color photocopies are deposited in the University of Idaho Herbarium (ID) as facsimile vouchers, along with additional collections from the same populations.

Morphometric analysis.—As a quantitative approach to capturing morphological subtlety, a morphometric analysis of characters that can be scored from herbarium specimens was made. Characters cited by authors in the original species descriptions were used whenever possible. Some characters that are valuable to botanists in the field, such as color, texture, or folding of pinnae, could not be scored because they are distorted or destroyed by pressing and drying. Forty-one different measurements or ratios were recorded for each plant. Measurements were made using a Panasonic WV-CD20 video camera and Mocha image analysis software (SigmaScan Pro version 3.0 Jandel Scientific).

Analysis.—Canonical discriminant analysis identifies one or more canonical variables that are linear combinations of multiple measured characters. These canonical variables can show the greatest morphological differences between groups. Statistical calculations were performed using the SAS CANDISC procedure, SAS Release 6.11 (SAS Institute Inc.), on the same samples of *B. minganense* and *B. crenulatum* used for genetic analysis, excluding three plants that were browsed. Two very unusual plants of *Botrychium minganense* also were excluded from the morphological analysis. One was extremely large, and the other had only rudimentary peg-like pinnae. One plant (cLapover.09) was excluded because it displayed an additive RAPD profile, and thus was possibly a hybrid. Preliminary one-way ANOVA showed that the means of many characters were significantly different between the species at the alpha = 0.05 level, including the ratio of trophophore width to the width of its axis (reflecting the tendency of pinna margins to be decurrent on the rachis); average angle of the margins of the four basal-most pinnae (degree of fanning); ratio of the length of the space between the first two pinnae pairs to greatest pinna width (a measure of overlapping of pinnae); ratio of greatest pinna width to least pinna width; ratio of length to width of trophophore; ratio of length to width of sporophore; ratio of pinna width to length; length of the sporophore; average angle made by the four basal-most pinnae with the rachis; total height (ground level to tip of sporophore); length of trophophore; length of trophophore stalk; and length of gap between first two pinna pairs. Measurements of these characters are illustrated in Figure 1. All pinna measurements were made from the same pinna for each plant, one of the largest pair. In the largest plants the lowest pinnae are sometimes partly transformed into sporangial branches. In that case one of the largest untransformed pair was chosen. The ratios of 1) trophophore width: trophophore axis width and 2) maximum:minimum pinna width were log-transformed, and the length of sporophore was square root-transformed to bring them closer to a normal distribution. The distribution of all variables used was judged to be within the limits of robustness of the procedures (K. Steinhorst, pers. comm.). Variances were compared between species groups for each character to see that they were equal, or if not, the variance of the larger group did not exceed that of the

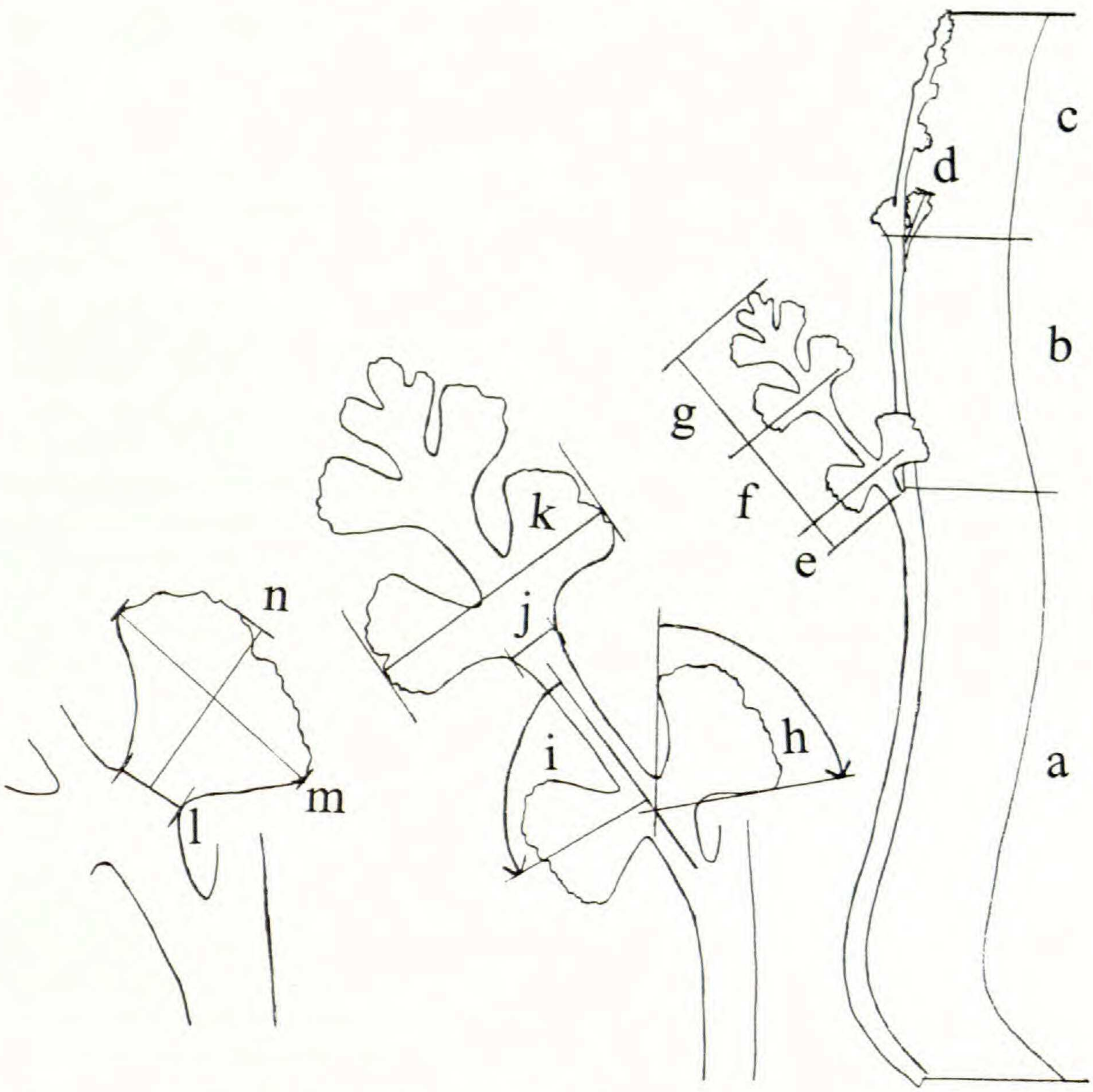

FIG. 1. Measurements made for characters that were significantly different between *Botrychium minganense* and *B. crenulatum* in morphometric analysis. a. Length of common stalk. b. Length of sporophore stalk. c. Length of sporangia-bearing part of sporophore (b+c = length of sporophore, a+b+c = total height. These and any other curved lengths were traced directly on the image of the plant). d. Length of longest sporophore branch (2d = sporophore width). e. Length of trophophore stalk. f. Distance between centers of first two pinna pairs. g. Balance of length of trophophore (e+f+g = length of trophophore). h. Angle of edges of pinna (for average of four basal-most pinnae). i. Angle at which pinna meets axis of rachis (for average of four basal-most pinnae). j. Greatest width of rachis. k. Trophophore width. l. Least width of largest pinna m. Greatest width of largest pinna. n. Length of largest pinna.

smaller group by more than a factor of 2.5, a conservative level chosen for unequal sample sizes. In general, variances were greater for *B. minganense*.

RAPD analysis.—DNA was isolated from 10 mg samples of each pressed plant. For those plants that were less than 10 mg, the whole plant was used. Plants were ground on ceramic well plates with liquid nitrogen, ground further

with 600 µl 70°C CTAB buffer, and transferred to 1.5 ml tubes. The grinding buffer and subsequent isolation procedures followed Stewart and Via (1993), with the following modifications: the homogenate was incubated at 70°C for 30 minutes before the chloroform extraction, the precipitated DNA pellet was washed with 1 ml cold 76% ethanol with 10 mM NH_4AC, and the dry pellet resuspended in 50 µl TE. Of several tested, this protocol was the least likely to yield gummy residues coprecipitating with the DNA, which was a problem with some samples. The residue, when it occurred, was removed by centrifugation before quantifying the DNA with a fluorometer. DNA was amplified (Williams et al., 1990) in 25 µl reactions containing 1× buffer (Promega M190A), 0.1 mM of each deoxynucleotide, 2 mM $MgCl_2$, 0.00005% bovine serum albumin, 5 pmols 10-mer primer (Operon), 10 ng genomic DNA, and 0.5 units *Taq* DNA polymerase (Promega), overlaid with 25 µl mineral oil. Samples were amplified in an MJ Research PT 100 thermocycler (44 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, with a final 5 min at 72°C). Products were electrophoresed in 1.5% agarose gels, visualized by UV illumination after staining with ethidium bromide, and imaged with AlphaImager v. 3.2 software. Populations were divided among multiple PCR runs, and a subsample was run multiple times to confirm repeatability of each band chosen. Bands were scored manually by comparison to standard size markers. Bands are designated by the name of the primer with the approximate size in base pairs as a subscript, e.g. B-11_{575}.

Primer screening.—Primers were screened against two samples each of *B. minganense* and *B. crenulatum*. Twelve primers (A-11, B-11, B-12, C-6, C-8, C-9, C-10, C-11, D-11, D-16, D-20, X-1) showing the best well-spaced bands polymorphic in one or both species were selected for the final data set. One hundred ninety-four plants were scored manually for presence or absence of 74 RAPD bands each. As more species and populations were added, fewer primers and bands within primers could be used because some new bands were close to the position of old bands or amplified with different intensity, making them difficult to score. Therefore, the scoring is conservative and reflects only minimum differences among all populations, whereas many additional differences that are not included in the data set are readily apparent among individuals and populations in the same gel.

Cluster analysis of RAPD data.—UPGMA cluster analysis was performed on RAPD data with NTSYSpc version 2.02 (Rohlf, 1997) using simple matching and Jaccard metrics.

Results

Morphometric analysis.—Optimal separation of the two species on a morphological basis requires consideration of multiple characters at once. The six variables in Table 2, when analyzed together, provided the greatest separation of the groups in this data set. The canonical variate analysis tested the null hypothesis that there are no differences between the two species based on the chosen variables. This hypothesis was rejected at the $p = 0.0001$ level, with

TABLE 2. Correlations and coefficients of the six variables that provided the greatest discrimination between *Botrychium minganense* and *B. crenulatum* in Canonical Discriminant Analysis.

Variable	Pooled Within Canonical Structure CAN1	Pooled Within-Class Canonical Coefficients CAN1
AVANGMAR	0.50	0.65
AVANGPIN	0.16	0.40
PWIDLEN	0.20	−0.18
MLSPORO	−0.18	−0.94
NTWIDAX	0.51	0.51
NPINMAMI	0.41	0.52

AVANGMAR=average angle of pinnae margins; AVANGPIN=average angle of pinnae with rachis; PWIDLEN=ratio of pinna width:pinna length; MLSPORO=square root transformed length of sporophore; NTWIDAX=log-transformed ratio of trophophore width greatest width of trophophore axis; NPINMAMI=log-transformed ratio of greatest pinna width:least pinna width.

$F = 35.52$ and degrees of freedom of numerator 6 and denominator 164. Canonical scores may be computed by taking the original value for each plant on each measurement, multiplying it by the respective canonical coefficient from CAN1 (Table 2), and adding all these products plus a constant adjustment for the means. Scores graphed by species form two overlapping groups, with the mean for *B. crenulatum* at 1.81 and the mean for *B. minganense* at −0.71 (Fig. 2). CAN1 scores of 55 of the total of 171 plants, or 32%, fell in the 0 to 2 range where species identity is ambiguous. Scores of 92 plants of *B. minganense* out of 123 (75%) fell in the 0 to −4 range, and 23 plants of *B. crenulatum* out of 48 (49%) scored from 2 to 4, where each had a high probability of correct species identity. Only one *B. minganense* had a CAN1 score above 2.

Canonical variates can be interpreted in terms of those variables that contribute the most to the separation of the groups. Although canonical variates are artificial and must be interpreted with caution, they can be identified in terms of their correlations with the original individual variables (Johnson and Wichern, 1992). These "within" structure coefficients indicate how closely a variable and the canonical variate are related, or the extent to which they carry the same information (Klecka, 1980). The ratio of trophophore width:trophophore axis width had the highest correlation, 0.51, followed by average angle of pinna margins, 0.50, and pinna maximum width:minimum width, 0.41. The within-class correlation for pinna width: length was 0.20, average angle of pinnae to rachis 0.16, and length of sporophore −0.18.

Another way of looking at the contributions of each individual variable within classes is by comparing coefficients that have been transformed so their standard deviations are equal to 1. These standardized coefficients then measure the relative contribution of each variable to the canonical variate score. As a relative measure, the standardized coefficient of each variable will change depending on the contribution of other variables. If two variables share

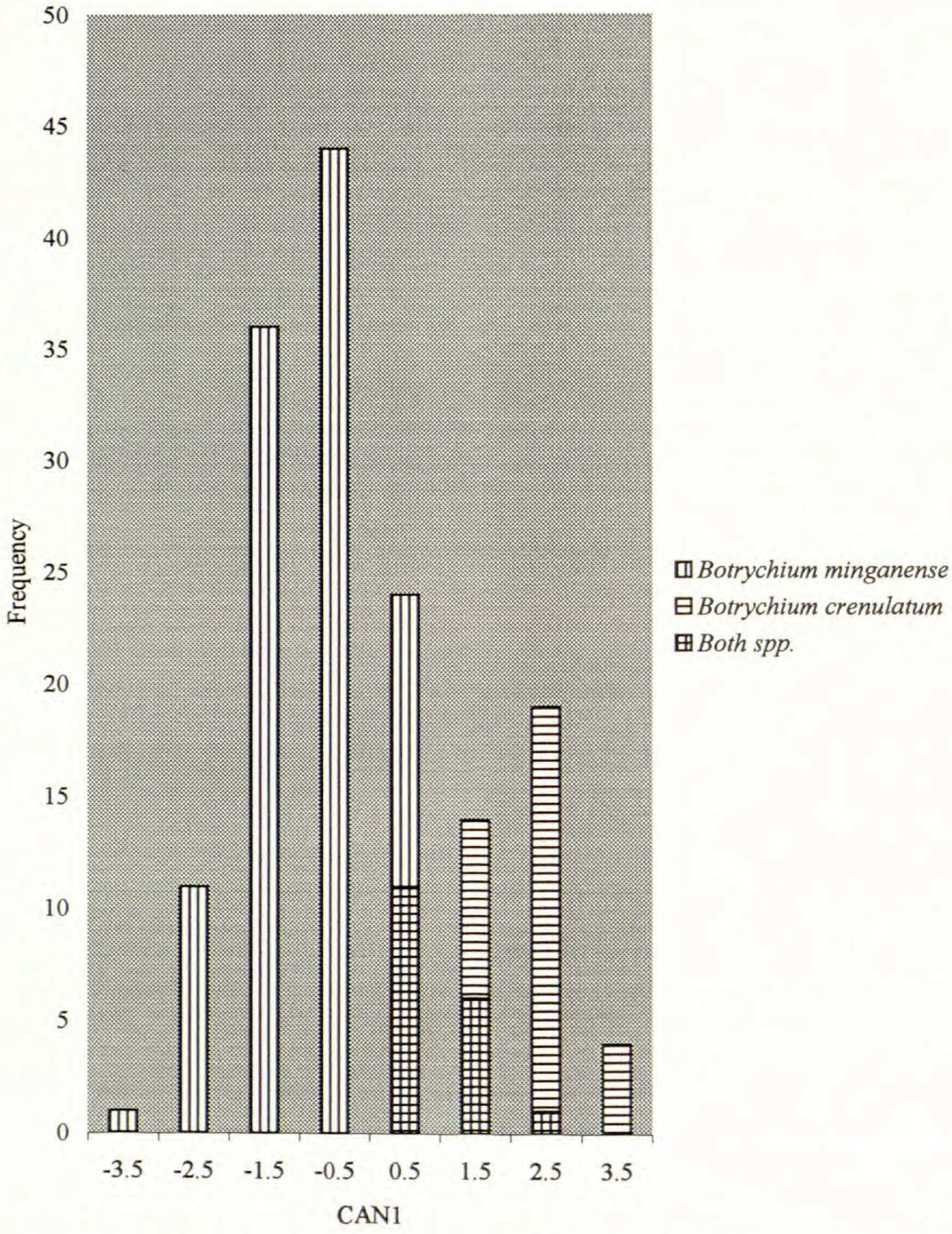

FIG. 2. Plot of CAN1 scores using six morphometric variables (Table 2) of 123 *Botrychium minganense* and 48 *B. crenulatum* plants whose species identity was confirmed genetically.

some of the same information (are highly correlated), the standardized coefficient value will be partly divided between them, but if one variable was not used, the standardized coefficient of the other would rise. They could also be larger but have opposite sign, so that one partially cancels out the other. The within structure coefficients, by contrast, are simple bivariate correlations

(Klecka, 1980). The standardized coefficient for length of sporophore, −0.94, had the highest absolute value among the variables. This was the measurement reflecting total size of the plant that contributed most to separation of the species. When it was included, other direct measures of size, such as total height, had small absolute values. Average angle of pinna margins, 0.65, and ratio of pinna maximum:minimum width, 0.52, are each related to the fanning of the pinnae in different ways, and they do not cancel each other out. The pooled within-class standardized canonical coefficient for ratio of trophophore width:trophophore axis width was also high, 0.51, and average angle of pinnae, 0.40, and ratio of pinna width:length, −0.18, were lower.

RAPD diagnostic bands.—*Botrychium crenulatum* was most differentiated (Table 3), set apart by seven bands (B-11_{1400}, C-6_{825}, C-8_{850}, C-8_{1700}, C-9_{1180}, C-10_{1275}, and D-11_{1075}) that did not occur in the other three species. One band (B-11_{1075}) was present in *B. crenulatum*, absent in *B. minganense* and *B. simplex*, and polymorphic in *B. lunaria*. One band (C-9_{1000}) was present in *B. crenulatum*, absent in *B. minganense*, and polymorphic in *B. lunaria* and *B. simplex*. Bands not present in *B. crenulatum* included six that were present in all individuals of *B. minganense*. Of these, three (C-11_{675}, D-16_{775}, and D-20_{890}) were polymorphic in *B. lunaria* and present in all *B. simplex*, two (B-11_{575}, C-9_{690}) were polymorphic in *B. lunaria* and absent in *B. simplex*, and one (D-11_{1225}) was present in *B. lunaria* and polymorphic in *B. simplex*. Four bands not present in *B. crenulatum* were present at high frequency (0.97–0.99) in *B. minganense*. Three of these (D-11_{875}, D-16_{400}, D-16_{510}) were polymorphic in *B. lunaria* and absent in *B. simplex*, and one (D-11_{1300}) was polymorphic in both *B. lunaria* and *B. simplex*. No bands in the sampled plants were unique to *B. minganense* or *B. lunaria*, and one band (C-6_{450}) was seen only in *B. simplex*. Bands common to all four species were not scored.

Clustering.—UPGMA clustering of the RAPD data using a simple matching metric resulted in four well-defined species groups (Fig. 3). The *B. crenulatum* cluster was most distinct. The *B. simplex* cluster and the *B. lunaria* cluster grouped together, and the "simplex lunaria" cluster associated most closely with the *B. minganense* cluster. Use of a Jaccard metric, discounting 0/0 matches, produced relationships conforming to those discussed below, except that the *B. lunaria* cluster associated most closely with the *B. minganense* cluster, and the *B. simplex* cluster grouped with the "minganense lunaria" cluster (dendrogram not shown). The *B. lunaria* and *B. simplex* clusters are displayed in Fig. 4.

Botrychium crenulatum.—Within the *B. crenulatum* group (Fig. 5), the largest cluster contained all the plants from Washington except two. This Washington cluster contained four subgroups within which plants had identical profiles. One subgroup included five OKCabin plants, and three contained plants from Deadman, cHodgson, and/or Aladdin. All *B. crenulatum* populations were polymorphic. Associated with the Washington cluster was a cluster that contained samples from Montana (Stewart) and Oregon (Lapover), plus two genetically distinct plants from the Hodgson population from northeastern Washington, which includes both *B. minganense* and

TABLE 3. Diagnostic RAPD bands.

Band name	*B. crenulatum*	*B. minganense*	*B. lunaria*	*B. simplex*
B-11_{575}‡#	0	1	P	0
B-11_{1075}**	1	0	P	0
B-11_{1400}***	1	0	0	0
C-6_{450}	0	0	0	1
C-6_{825}***	1	0	0	0
C-8_{850}***	1	0	0	0
C-8_{1700}***	1	0	0	0
C-9_{690}‡#	0	1	P	0
C-9_{1000}*	1	0	P	P
C-9_{1180}***	1	0	0	0
C-10_{1275}***	1	0	0	0
C-11_{675}‡▽	0[a]	1	P	1
D-11_{800}*	1	0	P	1
D-11_{875}†#	0	P(1)	P	0
D-11_{1075}***	1	0	0	0
D-11_{1225}‡○	0	1	1	P
D-11_{1300}†	0	P(1)	P	P
D-16_{400}†#	0[a]	P(1)	P	0
D-16_{510}†#	0	P(1)	P	0
D-16_{775}‡▽	0[a]	1	P(1)	1
D-20_{890}‡▽	0	1	P	1

1 = present, 0 = absent, P = polymorphic, P(1) = polymorphic, present very high frequency.
*** Present in *B. crenulatum*, absent in *B. minganense, B. lunaria, B. simplex.*
** Present in *B. crenulatum*, absent in *B. minganense*, polymorphic in *B. lunaria*, absent in *B. simplex.*
* Present in *B. crenulatum*, absent in *B. minganense*, polymorphic in *B. lunaria*, present or polymorphic in *B. simplex.*
‡ Present in *B. minganense*, absent in *B. crenulatum.*
† Polymorphic at high frequency in *B. minganense*, absent in *B. crenulatum.*
# Polymorphic in *B. lunaria*, absent in *B. simplex.*
▽ Polymorphic in B. lunaria, present in B. simplex.
○ Present in B. lunaria, polymorphic in B. simplex.
[a] Also present in Lapover9.

B. crenulatum. One of these individuals, "m"Hodgson.05, was classified in the field as *B. minganense*, but clearly groups genetically with *B. crenulatum*. The most distinct group was formed by Oregon plants. Goofy was the only population to form an exclusive cluster. Goofy and Lapover grouped together, but some members of Lapover also clustered with Stewart, the Montana population, in the mixed cluster. cLapover.09 was the most dissimilar member of the *B. crenulatum* cluster, and in fact displayed three bands otherwise found only in *B. minganense*, *B. lunaria*, or *B. simplex* (C-11_{675}, D-16_{400}, and D-16_{775}) as well as all the bands displayed only in *B. crenulatum*.

Botrychium minganense.—In the *B. minganense* group (Fig. 6), three populations formed exclusive clusters: Watson (Oregon), Poison (Oregon), and Devil (Washington). All others formed mixed groups. Thirteen plants from the Idaho Panhandle and neighboring Montana populations (Rock, Deer, and Kelsey) had identical profiles. Some members of each of those populations

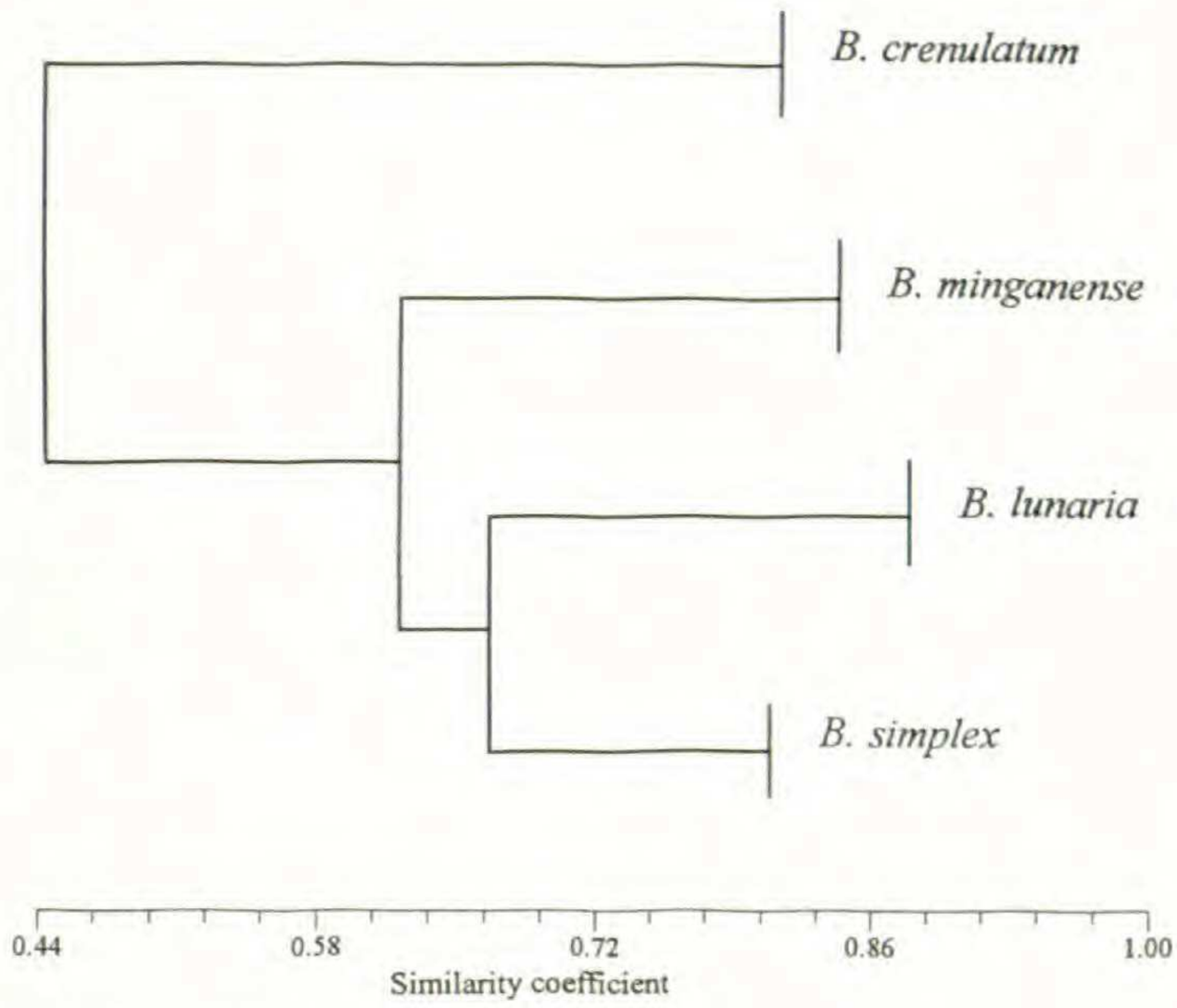

FIG. 3. Species clusters from UPGMA dendrogram of RAPD data from a total of 194 plants of four moonwort species: 49 *Botrychium crenulatum*, 128 *B. minganense*, 12 *B. lunaria*, and six *B. simplex*. The scale represents the similarity coefficient between clusters.

clustered with other groups. One population, Aladdin1, was monomorphic. Members of WenR and WenT, which grew adjacent to each other, each grouped in a separate larger cluster. Mill, from the Washington Cascades, and Manley, from northeastern Washington, were particularly diverse: each had members in four different larger clusters, and other members that were highly divergent.

DISCUSSION

Morphometrics.—The cryptic moonwort species *Botrychium minganense* and *B. crenulatum* can be separated by canonical variate analysis into two partially overlapping groups. Plotting the CAN1 scores of 171 plants whose identity had been genetically confirmed showed that 32% fell in the zone of overlap where the two species could not be separated using the characters scored. The characters that contributed most to the separation were measures related to pinna shape, proportions of the trophophore, and size. Pinna fanning, as reflected in the pinna shape characters, is emphasized in descriptions of *B. crenulatum* (Wagner and Wagner, 1981; W. H. Wagner, 1993). Average size of *B. minganense* is larger than that of *B. crenulatum* (mean height of sampled plants 84 mm and 73 mm respectively), but each can be less than a centimeter tall (pers. obs.). The ratio, width of trophophore: maximum width of trophophore axis, is a complex character that combines elements of several differences between the species, including length of pinnae, angle at which the pinnae meet the axis, and tendency of pinnae to be decurrent on the trophophore axis or of the axis to be flattened. These characteristics have not been emphasized in taxonomic descriptions of *B. minganense* and

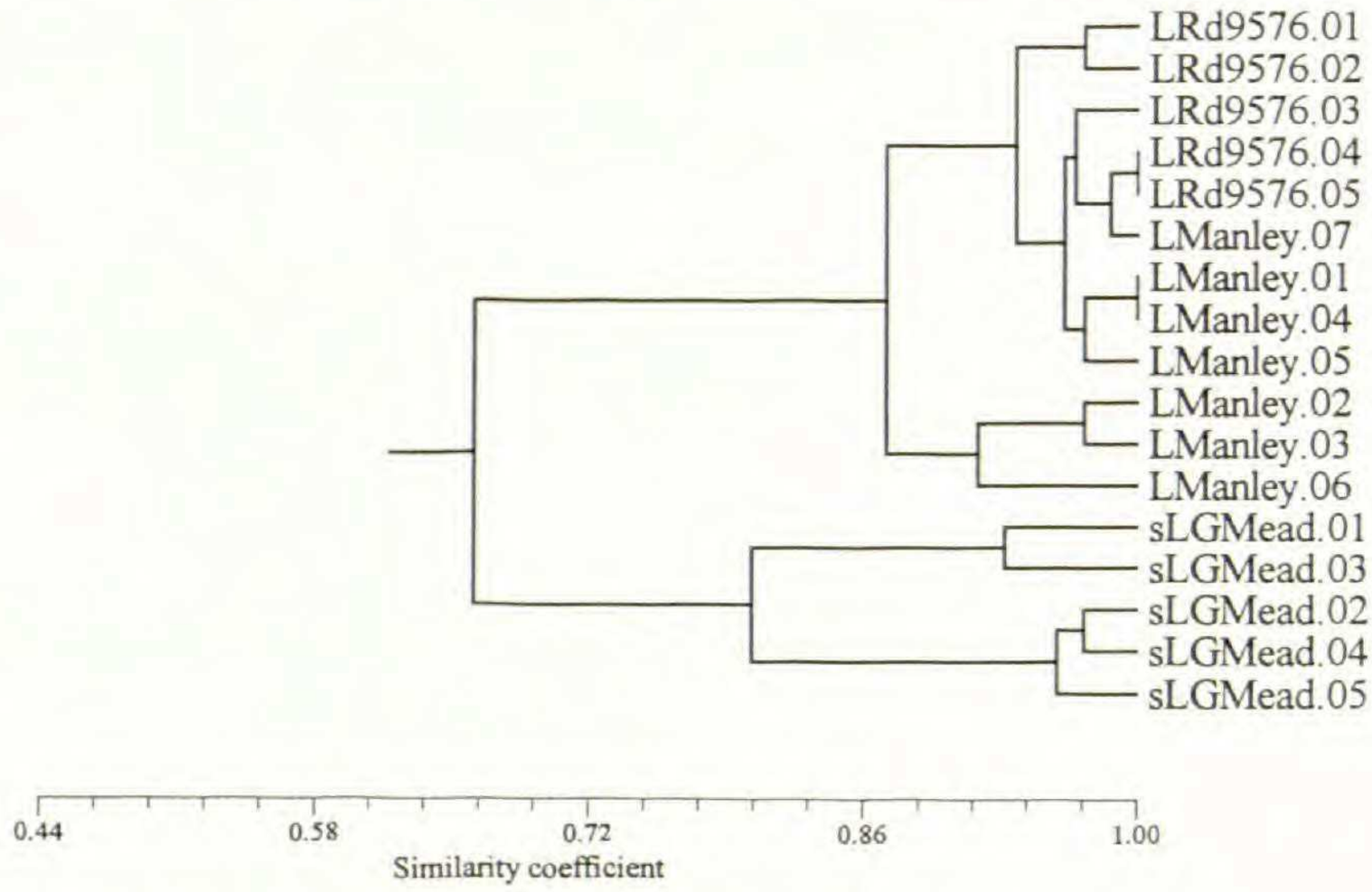

FIG. 4. Detail of *Botrychium lunaria* and *B. simplex* clusters from UPGMA dendrogram of RAPD data (Fig. 2). Plants are labeled by population abbreviation (Table 1) and individual number within population. Leading letter signifies species (L = *B. lunaria*, s = *B. simplex*). The scale represents the similarity coefficient between clusters.

B. crenulatum, although the trophophore of *B. minganense* has been described as narrow (Wagner and Wagner, 1993).

The statistical analysis of morphology was limited compared to field identification. Some useful morphological characters of live plants could not be used in an analysis of herbarium specimens. The characters that could not be captured include phenology, color, texture, and many aspects of plant habit, such as cupping of the pinnae. Other useful characters, such as the number of pinna pairs, or number of crenulations on pinna margins, are not normally distributed and therefore violate the requirements for canonical variate analysis.

The 32% ambiguity rate in the morphological analysis contrasts with the correct field identification of all but seven of the 171 analyzed plants. However, in the field, individual plants are not independently identified, as is the case with the statistical analysis. Field botanists generally examine the range of variation at a site and make an identification on the basis of a group of typical plants. Some well-developed plants will show characters that smaller ones lack. This is also true of herbarium identification. In fact, consulting botanists request collections of about a dozen plants for identification of moonwort species (Wagner, 1992; Wagner and Wagner, 1993; Zika et al., 1995). This study generally supports the assumption that similar plants associated at one site belong to the same species. In the genetic analysis, none of the populations identified in the field as containing *B. minganense* and not *B. crenulatum*, or *B. crenulatum* and not *B. minganense*, contained sampled individuals of the other species. However, even experienced botanists can occasionally be misled (example given in Farrar, 1998). Although we sought mixed populations for this study, only three of 23 (Hodgson, Manley, Lapover) had mixed *B. minganense* and *B. crenulatum* populations.

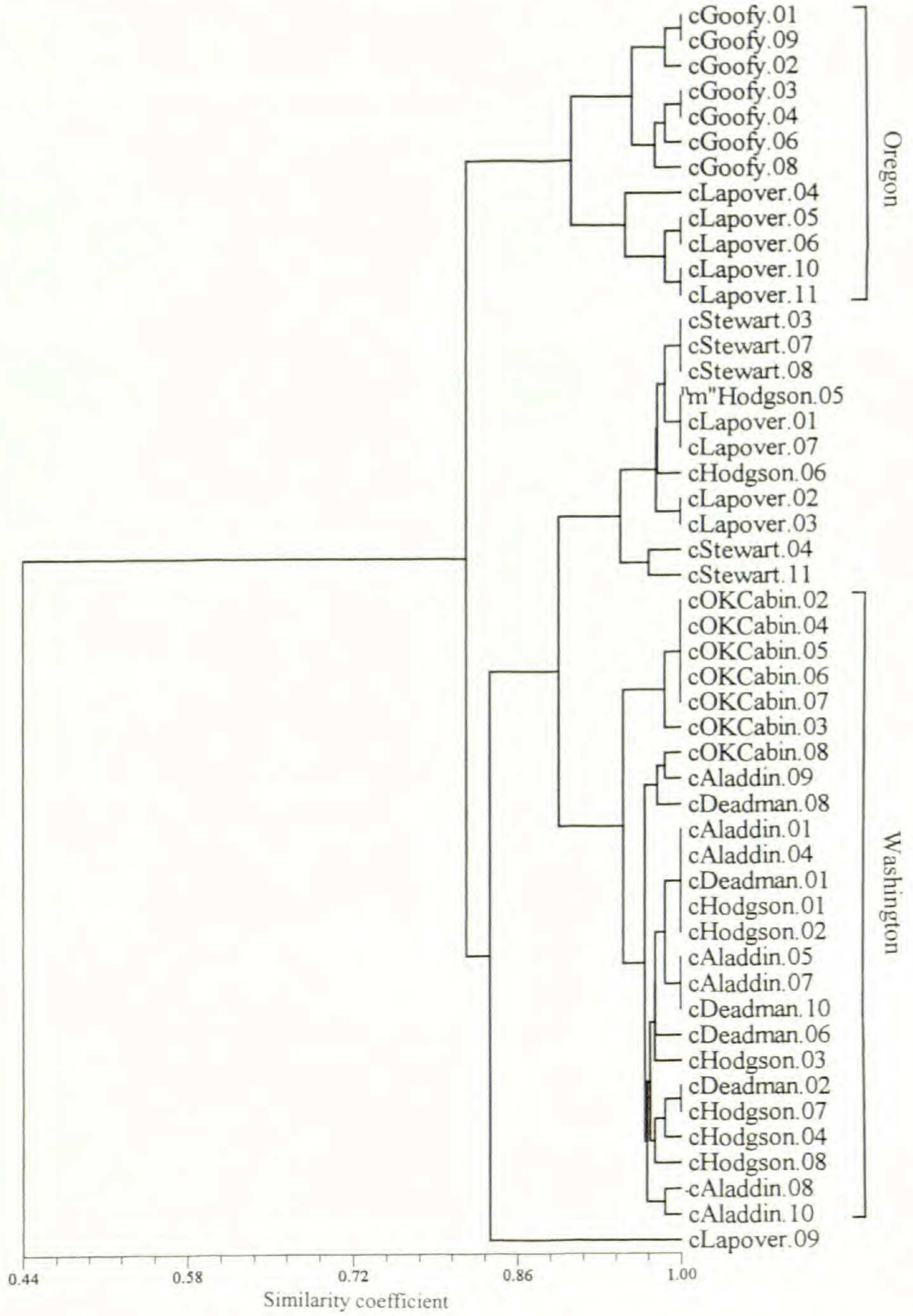

FIG. 5. Detail of *Botrychium crenulatum* cluster from UPGMA dendrogram of RAPD data (Fig. 3). Plants are labeled by population abbreviation (Table 1) and individual number within population. Leading letter signifies species as identified in the field (c = *B. crenulatum*, "m" = *B. minganense* as identified in the field). The scale represents the similarity coefficient between clusters.

RAPD analysis: Interspecific variation.—In contrast to the morphometric results, all sampled plants, with one exception (cLapover.09), grouped clearly by species based on RAPD markers. Primers C-10 and/or D-11, run with a known *B. crenulatum* sample, would be sufficient to confirm or rule out the identification of *B. crenulatum*. Separation of *B. minganense* from *B. lunaria* by means of RAPD markers requires more primers; because neither has unique bands and they are separated in the similarity analysis by differences in

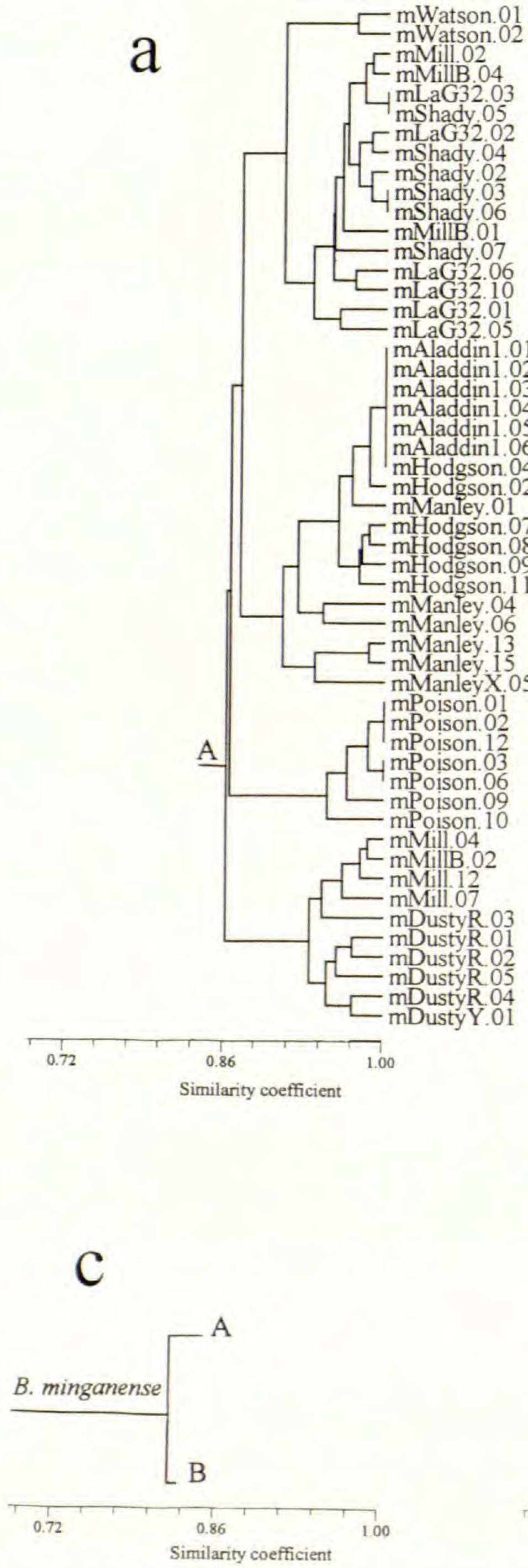

FIG. 6. Detail of *Botrychium minganense* cluster (c) and subclusters (a, b) from UPGMA dendrogram of RAPD data (Fig. 3). Plants are labeled by population abbreviation (Table 1) and individual number within population. Leading letter signifies species as identified in the field (m = *B. minganense*). The scale represents the similarity coefficient between clusters.

frequencies of bands. All *B. simplex* sampled had one band that appeared to be unique to the species, but more populations must be sampled to verify this as a species marker. The assignment of plants to species based on RAPD markers agreed with their classification in the field, with one exception. That plant, "m"Hodgson.05, had a CAN1 score of 0.88 in the morphometric analysis (Fig. 2) and fell in the range of minganense/crenulatum overlap. It was field-identified as *B. minganense*, but had the RAPD pattern of *B. crenulatum*. It was a small plant with non-crenulated pinnae, from a mixed population of *B. minganense* and *B. crenulatum*.

RAPD analysis: Distribution of variation within species.—RAPDs provide greater evidence of variability in species of *Botrychium* than isozymes have. Hauk and Haufler (1999) reported 14 isozyme genotypes among 252 plants of *B. minganense*, and one genotype among nine plants of *B. crenulatum*. In this study, there were 100 RAPD genotypes among 128 individuals of *B. minganense*, and 28 genotypes among 48 plants of *B. crenulatum*. Within five populations of *B. minganense* (Watson, Bulldog, mHodgson, LaGrande32, and Dusty) and one of *B. crenulatum* (Deadman), no two sampled individuals had the same RAPD profile.

The population showing the highest genetic similarity among individuals was *B. minganense*–Aladdin1. This small population of about ten plants growing in approximately 9 m^2, was sampled heavily because it was morphologically ambiguous, and could not be identified to species in the field (W. H. Wagner, Jr., pers. comm.). The plants were small, light green, and displayed rather broadly fanned pinnae. Three of the sampled plants had CAN1 scores in the 0 to 2 range where the scores of species groups overlapped, and three had slightly negative scores in the "minganense" range. This was the only population that lacked within-population variation (scorable or unscorable) on the gels, and might be described as clone-like. All other sampled *Botrychium* populations were larger, and had more than one RAPD profile.

Most individuals from the Deer (Idaho), Rock (Idaho), and Kelsey (Montana) populations of *B. minganense* had the same RAPD profile. These populations were located within approximately 50 km of each other. However, proximity was not a good predictor of genetic similarity across all populations. The twin *B. minganense* populations from Wenatchee Ford, in Washington, grew about 30 m apart and were reported as one population on the Wenatchee Forest sensitive plant sighting form. They were kept separate in the analysis because one group was growing in deep shade in a riparian zone under *Thuja plicata* and *Tsuga heterophylla*, and the other was under *Rubus parviflorus* on a roadside. The *Tsuga* group clustered with the Mill population from a mountain meadow on the Wenatchee Forest, but the *Rubus* group clustered with Flowery and Bulldog, shaded forest sites from northeastern Washington, approximately 225 km away. No association between ecological sites and RAPD genotype is evident in this or other clusters.

Although some populations, such as Goofy (*B. crenulatum*) and Devil (*B. minganense*), showed low similarity to any other, most populations had members in more than one cluster. For example, some *B. minganense* plants

from Mill clustered with plants from Manley in northeastern Washington, whereas others clustered with Shady and LaGrande32 in northeastern Oregon, about 400 km from the Mill site.

Genetic variation within *B. minganense* did not suggest any coherent genetic groups that might be associated with its morphological variability. Hauk and Haufler (1999) reported more isozyme variability within *B. minganense* than any other polyploid sampled. Given that RAPDs are revealing more variability than isozymes, additional sampling from across the species range may reveal genetic patterns within the species.

Genetic structure of populations.—The contrasting patterns of genetic similarity may result from processes that hinder or promote genetic isolation. Two important factors influencing the structure of genetic variation in plants are breeding system and dispersal of propagules. The breeding system of moonworts is not known from experimental investigations, because they have not been cultivated successfully. The most direct evidence comes from the allozyme work of Hauk and Haufler (1999) on other species of subgenus *Botrychium*. Low variability within populations hampered their inferences of breeding systems, but they attributed the low frequency of heterozygotes found in four populations of diploid moonworts (*B. simplex* and *B. lanceolatum*) to inbreeding. Electrophoretic studies on *Botrychium* species in subgenus *Sceptridium* (McCauley et al., 1985; Watano and Sahashi, 1992) and subgenus *Osmundopteris* (Soltis and Soltis, 1986) reported extremely high levels of inbreeding. Outcrossing may be hindered by the underground gametophytes of this genus (Tryon and Tryon, 1982), although moonwort hybrids have been reported (Ahlenslager and Lesica, 1996; Wagner, 1980, 1991; Wagner et al., 1984; Wagner, Wagner, and Beitel, 1985; Wagner and Wagner, 1988), demonstrating at least occasional outcrossing. The number of allopolyploids also documents that outcrossing is an important evolutionary process in subgenus *Botrychium* (Hauk and Haufler 1999). In our study, the lack of genetic diversity in the RAPD profiles of the small Aladdin1 population is consistent with inbreeding.

Genetic variability within populations of an inbreeding species could be increased by immigration of propagules from distant sources, and occasional outcrossing. Fern spores are light and can travel long distances, as ferns colonize remote islands (Tryon, 1970; Tryon, 1986; Ranker et al., 1994). Tryon (1970) presented evidence that 800 km is not a significant barrier to the migration of a fern flora. Tryon and Tryon (1982) characterized Ophioglossaceae in particular as a colonizing group.

Because of the dominant inheritance of RAPD markers (Bachmann, 1997; Crawford, 1997), these data do not provide unequivocal insights into the breeding system of moonworts. Although the variability detected in this study may not have been predicted based on isozyme studies, it is not inconsistent with a high dispersal rate and a largely inbreeding mating system.

Ancestry of B. minganense.—The *rbcL* sequence of tetraploid *B. minganense* did not match that of any known diploid (Hauk, 1995). On the basis of the match between the hypothetical isozyme profile of the non-chloroplast parent

of *B. minganense* and the isozyme profile of *B. crenulatum*, Hauk and Haufler (1999), proposed *B. crenulatum* as the most likely candidate for that parent. The RAPD data, however, did not support this relationship, because *B. crenulatum* showed seven unique bands absent in *B. minganense*. An earlier hypothesis (F. S. Wagner, 1993) based on morphological data, proposed *B. lunaria* and *B. pallidum* as the parental diploids. The RAPD evidence is consistent with a close relationship between *B. minganense* and *B. lunaria*, because neither had bands that did not occur in the other. More genetic evidence is needed to clarify the origins of *B. minganense*.

cLapover.09.—The identity of one plant from the Lapover site in the Lostine River Valley, Oregon, was uncertain when it was collected. It combined the color and luster of *B. crenulatum* with rounded, broad-based pinnae otherwise seen only in *B. minganense*. The RAPD profile of this plant included all seven diagnostic *B. crenulatum* bands, plus three characteristic *B. minganense* bands including C-11_{675} and D-16_{775} (also polymorphic in *B. lunaria* and present in *B. simplex*), and D-16_{400} (polymorphic in *B. lunaria* and not present in *B. simplex*). Eight bands documented in all sampled *B. minganense* were not present in the plant. cLapover.09 appears to be a hybrid, both because of intermediate morphology and mixed markers. The three "minganense" markers could also have come from *B. lunaria* or *B. simplex*, but these species were not recorded from the site, whereas *B. minganense* and *B. crenulatum* were present. Other moonwort species recorded at the site were *B. ascendens* and *B. lineare*, for which we have no RAPD data. Neither of these moonworts typically has rounded pinnae. Because not all of the diagnostic *B. minganense* bands were present, cLapover.09 does not appear to be an F_1 hybrid between *B. minganense* and *B. crenulatum*, but is more likely a backcross or later generation hybrid derivative.

Conclusions

Although many plants of *B. minganense* and *B. crenulatum* could not be reliably distinguished by canonical variate analysis of morphology, all sampled plants, except an apparent hybrid, could readily be assigned to species on the basis of RAPD profile. This supports the distinctness of the two species although their morphologies intergrade.

Although breeding system cannot be inferred from dominant markers such as RAPDs, higher levels of variability were detected within populations and species than might be predicted from previous genetic data, which suggested a high level of inbreeding. Thus codominant DNA markers such as microsatellites might be a productive avenue for further research into breeding system and evolutionary processes in *Botrychium*.

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